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Rapid Report

Depth profiling of dibucaine in sarcoplasmic reticulum vesicles by fluorescence quenching

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Abstract

The location of molecules of the local anesthetic dibucaine in sarcoplasmic reticulum vesicles (SRV) was determined using the quenching of its intrinsic fluorescence by iodide and by nitroxide-labeled stearic acids (SASL) with the nitroxide group at different positions of the fatty acyl chain. The molar ratios of dibucaine to Ca^{2+}-ATPase in the samples were less than 1. The acid-base titration of membrane bound dibucaine revealed a pK of 9.1, showing a negligible shift upon binding. The quenching data were obtained at pH 6.8 and are therefore related to protonated dibucaine. Quenching by iodide showed SRV-bound dibucaine to be more protected from collisions with iodide anion than dibucaine in buffer or even in neutral micelles. This shows the influence of negatively charged lipids in keeping iodide away from the ionic diffuse layer of the membrane surface where the dibucaine tertiary amine might be located. Analysis of the SASL quenching data indicates that dibucaine molecules are at a shallow position in the membrane bilayer. Their average depth was found to be at most that of the fourth carbon atom of the fatty acyl chain. The results do not exclude a preferential site for dibucaine in Ca^{2+}-ATPase, but if there is such site it must be located at the protein/lipid interface.

Key words: Sarcoplasmic reticulum vesicle; Dibucaine; Local anesthetic; Anesthetic; Fluorescence

An important question related to the investigation of the molecular mechanisms of anesthetic action is the nature of the final target of anesthetic molecules. The amphipathic character of substances having anesthetic properties gave rise to investigations of alterations induced on the organization of lipids in membranes [1,2]. Accumulating evidences of local anesthetics interactions with some membrane proteins [3,4] point to hydrophobic sites on specific proteins, mainly ionic channels related to the propagation of nervous pulses, as the final target of anesthetic action. On the other hand, since the hydrophobic domains of integral membrane proteins are inserted in the lipid bilayer, lipids appear to be an important intermediate target to anesthetic action.

The chemical structure of many local anesthetics involves a tertiary amine and aromatic rings. The tertiary amine occurs in both charged and uncharged forms upon interacting with various biological systems. Some lines of evidence attributed the anesthetic action to the charged form [5], but there are indications that both forms are neurologically active [6,7]. The aromatic rings are responsible for rich fluorescence properties, which provide a natural probe for studying anesthetic interactions. For dibucaine, the fluorescence properties in aqueous solution and in micelles were obtained [8,9]. Experimental investigation has been done on lipid vesicles [10–12], and in more natural systems like integral protein containing membranes [13].

Fluorescence quenching has been extensively used for the analysis of biomembrane structure [14]. The short range fluorescence quenching by spin-labels have been used to estimate membrane penetration depths of fluorophores in membranes [15,16].

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Abbreviations: SRV, sarcoplasmic reticulum vesicles; n-SASL, spin-labeled stearic acid with a doxyl group at the nth carbon atom of the acyl chain; CTAC, cetyl trimethylammonium chloride; LPC, lysolecithin; SDS, sodium dodecylsulphate.

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Our aim in this work is to investigate the location of dibucaine molecules in a natural membrane system consisting of sarcoplasmic reticulum vesicles. Ca^{2+}-ATPase is responsible for more than 80% of the protein weight of these membranes, and its activity is perturbed by local anesthetics [17,18]. Steady-state fluorescence of dibucaine is used allowing experiments at low anesthetic concentrations, which is important to obtain information on the prime site of the anesthetic molecules. We use the aqueous solute quencher iodide (NaI) to probe the accessibility of bound dibucaine to ions in the aqueous medium. Since stearic acids intercalate in the lipid portion of the membranes we use spin-labeled stearic acids with the nitroxide group at five different positions of the fatty acyl chain as quenchers of dibucaine fluorescence to obtain a depth profile of this anesthetic in the bilayer.

Sarcoplasmic reticulum vesicles (SRV) containing more than 80% of light vesicles prepared from rabbit skeletal muscle [19,20] were supplied by L. de Meis (Biochemistry Department, Universidade Federal do Rio de Janeiro) and stored in liquid nitrogen. Dibucaine hydrochloride was purchased from Sigma. Spin-labeled stearic acids with the nitroxide groups at several positions of the fatty acyl chain (n-SASL with $n = 4$, 5, 8, 12, and 16) were supplied by A. Watts (Biochemistry Department, University of Oxford). All other reagents were analytical grade.

All the experiments were performed at room temperature (22°C). SRV suspensions in 0.067 M borate/citrate/phosphate buffer, with a protein concentration of 3.6 mg/ml were used throughout the work. This corresponds to a Ca^{2+}-ATPase concentration of the order of $3 \cdot 10^{-5}$ M, and a lipid concentration of the order of $3 \cdot 10^{-3}$ M. Dibucaine was added to the suspensions to a final concentration of $2 \cdot 10^{-5}$ M. Since dibucaine has only moderate affinity for membranes it is important to estimate the degree of binding to vesicles. The apparent binding constant of dibucaine to DMPC at 35°C and pH 7.0 was found to be about $2 \cdot 10^3$ M^{-1} [10]. Assuming this binding constant of dibucaine to SRV lipids there will be about 85% of dibucaine-bound molecules under our experimental conditions. Considering that our experiments were performed at a lower temperature and that SRV surface has a net negative charge, we would expect the binding constant to be even greater.

The pH values were obtained with a Corning 130 pHmeter equipped with a glass Ag/AgCl semimicro combination electrode. For the pH titrations pH adjustments were done by addition of aliquots of 1 M HCl solution. Absorption measurements were performed on a Shimadzu UV-180 spectrophotometer. Fluorescence measurements were performed on a Jasco FP-777 spectrofluorometer. Dibucaine fluorescence was excited at 328 nm (maximum of the lowest energy absorption band) and the whole emission band was detected. Quenching experiments were performed using concentrated solutions of quenchers, sodium iodide (1 M), n-SASL (14 mM to 120 mM in ethanol), to titrate the dibucaine-SRV suspensions. Small aliquots of the solutions of quenchers were added to the samples using Hamilton microseringes. After each addition of stearic acids the samples were gently stirred and allowed to equilibrate until no changes in the fluorescence intensity was observed (10 to 20 min). The concentrations of spin-labeled stearic acids were measured on a EPR Varian E9 spectrometer, comparing the integrated intensity of the signals obtained with diluted solutions of labels with that of a standard solution prepared from a sample of 5-SASL from Sigma.

Membrane-bound molecules frequently present a displacement in the acid-base equilibrium of their ionizable groups. The fluorescence emission of dibucaine is sensitive to its ionization state [8,9] and is used here to study its acid-base dissociation in sarcoplasmic reticulum vesicles. The peak of fluorescence spectrum of dibucaine is blue shifted (from 411 to 395 nm at pH lower than 7) upon addition of vesicles, indicating binding to sites of lower polarity than water. The integrated intensity of the emission band decreases with increasing pH due to the decrease of the quantum yield of neutral relative to charged dibucaine.

Fig. 1 shows the pH dependence of the integrated intensity of the fluorescence band of dibucaine in buffer and in the presence of SRV. The value for the apparent pK (pK_a) of dibucaine obtained from the curve in Fig. 1 is 9.1. This value is very similar to that in buffer (9.0). This can be explained by two opposing effects: the lower dielectric constant of the bilayer, which causes a downward shift of pK, and the electric potential due

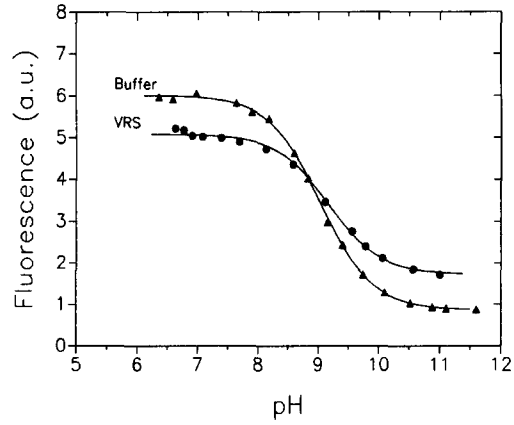

Fig. 1. Fluorescence of dibucaine (integrated over the emission band) in 0.067 M borate/citrate/phosphate buffer in the absence and presence of 3.6 mg/ml sarcoplasmic reticulum vesicles as a function of pH; the lines are best fits for the data, using the equation for the acid-base equilibrium, with pK values of 9.0 and 9.1, respectively.

to negatively charged lipids, which shifts the apparent pK to higher values.

These results show that in SRV at pH 6.8 there are more than 99% of dibucaine molecules in the protonated form.

Iodide is an anion and do not penetrate the hydrophobic core of membranes. For this reason sodium iodide was used as a quencher of dibucaine fluorescence to probe the accessibility of SRV bound dibucaine to the ions in the aqueous phase.

Quenching by NaI was measured for dibucaine in the absence and presence of SRV (3.6 mg/ml of protein). The results are presented in Fig. 2, together with previous results [9] for the quenching of dibucaine fluorescence in micellar systems of different charge. The slopes of the plots give the Stern-Volmer quenching constants K_{SV}. The K_{SV} values obtained from the data in Fig. 2 are:

System	SRV	buffer	CTAC (cationic)	LPC (zwitterionic)	SDS (anionic)
K_{SV} (M^{-1})	11	40	80	22	2.2

The Stern-Volmer constant for SRV-bound dibucaine is greater than that for dibucaine bound to anionic micelles, but it is smaller than that for zwitterionic micelles. SRV-bound dibucaine is more protected from the iodide anion than in zwitterionic micelles. This shows the influence of the negatively charged headgroups of lipids in protecting dibucaine from iodide.

Barghouthi and Eftink [12] studied the quenching of dibucaine fluorescence by KI in the absence and presence of DMPC-DMPG vesicles. Dynamic quenching constants of 48 M^{-1} and 2.2 M^{-1}, respectively, were found. Our value in buffer is 40 M^{-1}, and the difference due to the difference in temperature. For dibucaine bound to SRV our value of 11 M^{-1} is likely to be slightly overestimated, because we neglected the fraction of dibucaine in solution, but since the degree of binding is high, as indicated by the estimations in Materials and methods and by the blue-shift of the fluorescence spectrum, corrections would certainly lead to values greater than 2.2 M^{-1}.

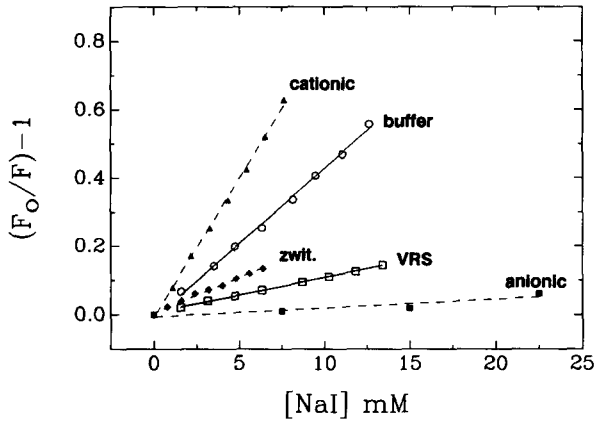

Fig. 2. Quenching of dibucaine fluorescence by iodide in borate/citrate/phosphate buffer in the absence (buffer) and in the presence of sarcoplasmic reticulum vesicles (SRV) pH 6.8, and ionic micelles of CTAC (cationic), LPC (zwiterionic), and SDS (anionic), at pH 5.7. The data for the ionic micelles were published elsewhere [9].

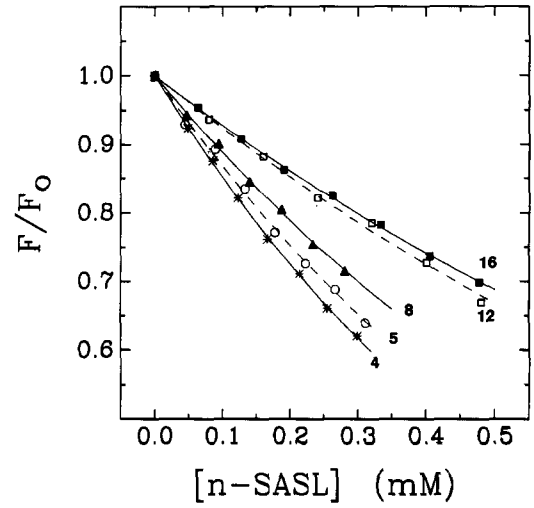

Fig. 3. Quenching of dibucaine fluorescence in sarcoplasmic reticulum vesicles by spin-labeled stearic acids with the nitroxide group at the nth carbon atom of the fatty acyl chain, with n = 4, 5, 8, 12, and 16. The lines are best-fit exponentials forced through the point (0,1).

Contrary to iodide, the stearic acids have a very high micelle/water partition coefficient and the number of molecules in the aqueous solution is negligible [21]. Using spin-labeled stearic acids with the nitroxide group at different positions of the chain to quench dibucaine fluorescence it is possible to determine the depth of a fluorophore in the lipid bilayer.

It is worth noting that, since the experiments were performed at a dibucaine concentration of 20 μM, and a Ca^{2+}-ATPase concentration of the order of 30 μM in the SRV suspensions, there is less than one dibucaine molecule per Ca^{2+}-ATPase molecule in the samples. The results for the quenching of dibucaine fluorescence by the n-SASLs appear in Fig. 3. Assuming static quenching and neglecting unbound dibucaine molecules, the data were fitted by exponentials forced through origin.

The static quenching constants, V, obtained fitting the data in Fig. 3 using exponentials (lines in the figure) are:

n	4	5	8	12	16
V (M^{-1})	$1.61 \cdot 10^3$	$1.43 \cdot 10^3$	$1.19 \cdot 10^3$	$0.81 \cdot 10^3$	$0.75 \cdot 10^3$

It is worth noting that these constants refer to the concentrations of the stearic acids relative to the volume of the suspensions. The local concentrations in the membrane are much greater (about 500-fold, con-

sidering a sample of 2 mg/ml of lipids with a density similar to that of water). The 'local' static quenching constants are therefore about 500-fold smaller.

4-SASL is more effective in quenching dibucaine fluorescence than any other of the deeper quenchers. Therefore, dibucaine molecules are at a shallow location in the membrane bilayer. The average depth is at most that of the fourth carbon atom of the fatty acyl chain.

Chattopadhyay and London [22] derived an equation to estimate the distance between the fluorophore and the shallowest quencher using a version of Perrin quenching equation. The equation is applicable to static quenching by a random distribution of quenchers in the plane of the membrane. The use of a static model was justified by the fact that the quenching processes in membranes are static in many cases and even for dynamic quenching diffusion in membranes is slow.

We used the static quenching constants above as an attempt to estimate the distance between dibucaine and the shallowest label by Chattopadhyay and London's equation. The results were, however, not unique, depending on the pair of quenchers used in the calculations, and did not add more information than the one obtained above. We attribute the failure in obtaining the same values for all label pairs to the deviations from a pure static quenching process and to the fact that the depth of the quenchers do not increase linearly with the chain length.

Fluorescence quenching caused by spin labels are short range [15]. The degree of quenching of dibucaine fluorescence by the spin-labeled stearic acids has the same order of magnitude as other liposome solubilized fluorophores, like porphyrins [22]. On the basis of the accessibility of dibucaine in SRV to the quenchers in the different depths of the membrane, the existence of a highest affinity site for dibucaine in the extramembranous region of Ca^{2+}-ATPase or in the bulk polypeptide chain is improbable. We cannot discard, however, a site at the lipid protein interface.

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